Towards novel biomarkers and rational nutritional interventions in Inflammatory Bowel Disease

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SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

In **Chapter 1**, a general introduction about Crohn's disease (CD) is presented, with an emphasis on the role of the gut microbiota. In addition, we introduce the concept and importance of *in vitro* systems to study host microbe interaction in health and disease. Moreover, we introduce the relevance for biomarkers in predicting inflammatory disease activity. Finally, we describe the background for initiating a clinical study to investigate the effect of a nutritional therapy on several parameters of CD disease outcomes.

In **Chapter 2**, we provide an overview of recently developed co-culture models for studying host-microbe interactions. We discuss the pivotal role that gut microbiota play in both health and disease. Many diseases are associated with an aberrant gut microbiota composition. In particular, we focus on the role of *Faecalibacterium prausnitzii* in CD; since it is known that this bacterial species is significantly reduced in abundance in the bowel of CD patients. A distinction can be made in different laboratory model systems for studying host-microbe interactions. We describe three broad categories: **1).** Models for the gut epithelium and mucosa. **2).** Models for gut bacteria. **3).** Models for gut host-microbe interactions; the main subject of this review chapter. In further detail, we discuss five models belonging to the latter group: **3A).** Transwell co-culture models. **3B).** The Host-Microbiota Interaction (HMI™) module. **3C).** The 'Human oxygen-Bacteria anaerobic' (HoxBan) co-culturing system. **3D).** The Human gut-on-a-chip. **3E).** The HuMiX (human-microbial crosstalk) modular microfluidic device. We review the different systems in view of application for studying human host-microbe interactions, and provide a schematic overview of their advantages and disadvantages.

The Human oxygen-Bacteria anaerobic (HoxBan) system allows co-culture of strict anaerobic gut bacteria together with oxygen-requiring gut epithelial cells within one system. In **Chapter 3**, we introduce the first studies of this novel co-culture model, consisting of a 50 mL tube, filled with solidified YCFAG agar in the bottom 'bacteria' compartment (anaerobic), and the 'human' top compartment (aerobic), containing DMEM medium, in which the coverslip-attached epithelial cells are placed upside down (cells facing the agar). We were able to co-culture the strict anaerobic bacteria *F. prausnitzii* together with oxygen-requiring Caco-2 cells (epithelial cell line) for up to 36 hours. These human intestinal cells promoted the growth of *F. prausnitzii* in the HoxBan system and *F. prausnitzii* did not compromise Caco-2 cell viability. Moreover, *F. prausnitzii* suppressed the expression of inflammatory and oxidative stress markers in Caco-2 cells as determined by qPCR, clearly demonstrating the anti-inflammatory capacity of this bacterium.

Any intervention in Inflammatory Bowel Disease (IBD) requires adequate monitoring of inflammatory disease activity. However, this is currently not available and the association between patient-reported symptoms and observed endoscopic inflammatory disease activity (gold standard) is poor. Because of the disadvantages of endoscopic evaluation (a high patient burden, procedure-related complications, such as bowel perforation or bleeding, and relatively high costs), non-invasive biomarkers are frequently used to early detect inflammatory disease activity (exacerbations) in IBD. Nowadays, fecal calprotectin is the most commonly used non-invasive biomarker in IBD but does unfortunately not adequately predict disease exacerbations. In **Chapter 4**, we aimed to identify potential novel inflammatory biomarkers (an array consisting of serum cytokines, chemokines and markers for angiogenesis and vascular injury) that might serve as additional biomarkers for inflammatory activity. For this study, we included 39 CD patients, subdivided into two groups according to fecal calprotectin (FC) levels ('normal' and 'increased' FC levels). Concentrations of inflammatory biomarkers were measured by performing an electrochemiluminescence (ECL) multiplex assay. We correlated these inflammatory biomarkers with FC levels, and found a highly significant positive correlation between the pro-inflammatory cytokines IFN-γ and CRP and FC levels. Furthermore, significant positive correlations between IL-6, TNF-β, SAA, IL-17A and FC levels were observed. Overall, this study shows a distinct cytokine profile in CD patients, that appeared to correlate well with an increased level of FC. Altogether, we demonstrate that a positive correlation exists between multiple serum Th1- and Th17-associated cytokines and the FC level, presenting additional non-invasive candidate biomarkers that could be of value in monitoring and treating disease activity in CD.

In **Chapter 5**, we evaluate a subset of 10 relevant inflammatory biomarkers in a larger cohort of 118 IBD patients (64 CD patients and 54 UC patients). In this follow-up study, we also included a group of 20 healthy individuals. From a subset of 71 IBD patients, we collected endoscopic results. In this study, we have demonstrated that six inflammatory biomarkers, consisting of cytokines, chemokines and acute-phase reactants (Eotaxin-1, SAA, IL-6, IL-8, IL-17A and TNF-α) all individually showed a better prediction of endoscopic disease activity than the currently used parameters to determine inflammatory disease activity (i.e. C-reactive protein, FC levels and clinical disease indices such as the HBI or SCCAI scores). Ultimately, a combined panel of Eotaxin-1, SAA, IL6 and IL-8 showed an optimal prediction of the actual mucosal status in IBD with a sensitivity of 90.7% and a specificity of 68.4%. This prediction model showed superior predictive accuracy as compared to routinely measured parameters that are currently used in the clinic.

In **Chapter 6**, we have analyzed the systemic redox status in CD as enhanced oxidative

stress is intimately associated with inflammation. Systemic redox potential might therefore be a biomarker of disease activity as well as a target for therapy. Our results show that albumin-adjusted plasma free thiols, as reflective measure of the systemic redox status, are strongly reduced in CD compared to healthy individuals. This result is even more remarkable since most of the CD patients in this cohort had clinical disease in remission (HBI < 5). In addition, we found that CD patients with solely colonic disease had significant reduced levels of free thiols in comparison with patients with involvement of the terminal ileum. Moreover, we demonstrate that thiols are inversely correlated with the inflammatory biomarkers CRP, SAA and IL-17A.

Impaired intestinal permeability (IP) has been suggested to have an important role in IBD. [2, 3, 4] IP biomarkers may have relevant clinical implications to predict disease activity in IBD.[5, 6, 7, 8] In **Chapter 7**, we have evaluated the potential application of enhanced permeability for assessing disease activity by applying the orally administered ^{52}Cr-EDTA permeability test as a non-radioactive alternative to assess IP in patients with CD. We were also interested in whether an association with the location of disease existed according to the Montreal classification (ileal, ileocolonic or colonic), and whether a link with key bacterial species in CD was present. In this prospective trial, we included 60 CD patients, from which 25 patients were considered to have low mucosal inflammation (FC < 100 µg/g) and 35 patients with increased inflammatory activity (FC > 100 µg/g). All patients ingested 20 mL of ^{52}Cr-EDTA (20 mmol/L) solution, where after 24-h urine was collected. Urinary chromium was determined with the highly sensitive and reproducible ICP-MS method and corrected for urinary creatinine levels. In this study, we found that patients with increased FC levels demonstrated elevated ^{52}Cr-EDTA excretion (though non-significantly). However, we were able to demonstrate a positive and significant correlation between ^{52}Cr-EDTA excretion and fecal calprotectin levels. Moreover, ^{52}Cr-EDTA excretion was most apparent in CD patients with colonic disease location (non-significantly). Lastly, ^{52}Cr-EDTA excretion negatively associated with relative abundance of *F. prausnitzii*, and a positive association with *Enterobacteriaceae* was observed.

As we found that an affected systemic redox status as well as a relatively low abundance of *F. prausnitzii* are key characteristics in CD patients, we decided to test whether riboflavin might have an effect on the outcomes of disease in CD. [1, 9] In **Chapter 8**, we present the results of the *RISE-UP* study. In this prospective intervention trial, we evaluated the effect of a riboflavin supplement in CD. At first, we assessed the effect of riboflavin on clinical and biochemical markers of disease activity. Moreover, we analyzed the gut microbiome composition. In total, 79 patients were included in this study. Nine patients were excluded for various reasons. The group of CD patients with a low inflammatory activity (FC level <

200 µg/g) at baseline consisted of 40 patients, the group with an increased inflammatory activity (FC level > 200 µg/g) consisted of 30 patients. We found that after riboflavin supplementation, clinical symptoms, as quantified with the validated Harvey-Bradshaw Index (HBI), improved. In addition, albumin-adjusted plasma free thiols increased, proving an antioxidant effect. The levels of serum inflammatory markers TNF-α and IL-2 showed a significant reduction. Also, commonly used parameters for the assessment of disease activity such as C-reactive protein (CRP) levels and the erythrocyte sedimentation rate (ESR) reduced significantly. Riboflavin supplementation led to decreased *Enterobacteriaceae* abundance in quiescent CD as determined by FISH. Using metagenomic shotgun sequencing (MGS), no significant effect was observed on either gut microbiota diversity, taxonomy and metabolic pathways. Finally, we demonstrated an enhancement in the Quality of Life (QoL), mainly in the subgroup of patients with normal FC levels.

DISCUSSION AND FUTURE PERSPECTIVES

HOXBAN CO-CULTURE SYSTEM

In recent years, much progress has been made in the field of gastro-intestinal *in vitro* research models. In order to gain more knowledge on the possible etiological role of gut-microbe interactions in the development of disease, the dysbiosis phenomenon as is characteristic for CD, should be further understood at a mechanistic level. Previously, major technological improvements to *in vitro* models have made it possible to co-culture obligate anaerobic bacteria and oxygen-requiring gut epithelial cells which is mandatory to adequately study host-microbe interactions. The HoxBan co-culture system is such an easy-to-use system for the fundamental analysis of bacterial-epithelial cell interactions and could be an attractive model to be used as screening model to gain more insight into the effects of dietary modulation on host-microbe interactions. Since clinical studies require a vast amount of time and entail many steps to reach to reliable conclusions, *in vitro* co-culture systems are very useful to precede translational dietary intervention trials. However, these models will always have their own limitations, since the gut ecosystem is extremely complex to mimic with an *in vitro* system. For instance, we recently optimized the system by improving the 'human' compartment of the system. Here, we implemented primary human intestinal epithelia, that are derived from isolation of gut-resident stem cells in intestinal crypts, creating 'gut organoids'. Caco-2 cells, that originate from human colorectal adenocarcinoma, are not fully representative of the human differentiated gut epithelium. In contrast, intestinal organoids contain all main types of epithelial cells as present *in vivo*, e.g. enterocytes, goblet cells, enteroendocrine cells and Paneth cells. Another advantage of these organoids is that it allows the researcher to customize the model to compare organoids of CD patients with organoids from healthy individuals in the HoxBan system. In addition, within CD patients, epithelial cells from different locations from the gut can be investigated (i.e. ileum or colonic disease) and signaling pathway's such as the HIF-1α pathway can selective be manipulated by CRISPR-Cas9 manipulation and analyzed by single cell RNA sequencing. Futhermore the immune component should be added to this system for instance by applying monocytes to the system and investigate the effect of different bacteria on monocyte activation, chemotaxis and immune activation.

This will not only provide detailed molecular insight in mutual host-microbiome interactions, but may also lay the foundation for personalized approaches to manipulate microbiome composition to improve human health. [10]

NOVEL BIOMARKERS IN IBD?

In IBD, non-invasive and reliable biomarkers are warranted to early detect inflammatory

activity of the intestinal mucosa. In addition, these biomarkers can be used to predict the response to a specific treatment, and can be used to evaluate long-term treatment outcomes.[11] In this thesis, we have evaluated the role of several potential biomarkers to detect inflammatory activity in IBD. We correlated potential interesting biomarkers with established markers of inflammatory activity in IBD. Currently, the most clinically relevant and established biomarker in IBD is fecal calprotectin (FC).

We have separately studied the systemic immune response in CD patients with a low and an increased FC level **(Chapter 4)** as biomarkers might be distinct in the two patient-groups. As discussed above, we observed a distinct cytokine profile, in which Th1- and Th17-associated cytokines (IFN-γ, CRP, IL-6, TNF-β, SAA and IL-17A) positively correlated with the FC level. A major limitation of this preliminary study, however, was that we did not have endoscopic results of this cohort. For this reason, we performed a successive study in which we resolved this main important limitation **(Chapter 5)**. In this follow-up study, we correlated a panel of relevant inflammatory biomarkers with endoscopic disease activity in a large cohort of IBD patients. We present a panel of four inflammatory biomarkers (SAA, IL-6, IL-8 and Eotaxin-1) that in combination showed high sensitivity and specificity in predicting mucosal status as determined with endoscopic evaluation. We think that these findings are very promising, since the predictive value of endoscopic mucosal activity was superior compared to serum CRP, FC levels and HBI/SCCAI scores. Treatment decisions are currently often made based on a combination of these clinical available parameters. Future research should focus on externally validating this proposed panel of biomarkers in another prospective cohort of IBD patients. If the predictive value of this panel of biomarkers remains superior to FC, this combined array of inflammatory biomarkers might be an attractive alternative to be used. Moreover, determining the cytokine profile in IBD patients may have particular relevance in determining the response to biological therapy. Novel biological therapies are all directed on the mucosal immune response, i.e. anti-TNF-α inhibition or IL-12 and IL-23 inhibition. Recently, we have already performed a study in which we correlated clinical response to a cell migration inhibitor vedolizumab with several inflammatory biomarkers. The preliminary results of this study highlight the diagnostic potential of inflammatory biomarkers to predict response to biological therapy.

In this thesis, we present plasma free thiols as a potentially novel biomarker in CD. We observed a significantly decreased concentration of plasma free thiols in CD patients. Moreover, within the CD cohort, free thiols were inversely related to CRP (indicative of a less favorable disease status) and several other inflammatory biomarkers. Since this study is the first to report on the role of free thiols in CD, more research is required to

confirm the observed effects. Future research should focus on comparing the systemic redox status within several stages of inflammatory disease activity, as evaluated by endoscopic results. Furthermore, free thiols as biomarker of systemic oxidative stress might play a role in detecting disease exacerbations and the prediction of therapy response. Ultimately, since plasma free thiols form a potential therapeutic target, antioxidant therapy might be a potential treatment strategy to improve disease status in CD. Also, it would be highly interesting to investigate the systemic redox status in patients with ulcerative colitis (UC) by quantifying the concentrations of plasma free thiols.

In this thesis, we show that in CD patients increased intestinal permeability, as reflected by high urinary ^{52}Cr-EDTA excretion, significantly correlates with fecal calprotectin levels, used as surrogate marker for inflammatory disease activity. Unfortunately, we were not able to make correlations between the ^{52}Cr-EDTA-measured intestinal permeability and endoscopic disease activity. Previously, ^{52}Cr-EDTA excretion had not been measured in a similar large and well-described cohort of CD patients as we presented. Our results indicate that there seems to be a correlation between inflammatory disease activity (with FC levels used as surrogate marker) and intestinal permeability (^{52}Cr-EDTA excretion) in CD. Furthermore, our study focused on using ^{52}Cr-EDTA instead of the radioactively labeled alternative, ^{51}Cr-EDTA, that has mostly been used in prior studies to evaluate intestinal permeability with a stable and inert complex as Cr-EDTA. Also, many of these prior studies used a gamma counter to quantify the urinary amount of ^{51}Cr. [12, 13, 14, 14] In our study, we quantified the amount of ^{52}Cr in the urine using ICP-MS. Future studies should focus on the potential utility of the ^{52}Cr-EDTA intestinal permeability test in relation to endoscopic disease activity. Furthermore, Cr-EDTA might be useful for the detection of the first stages of CD development, since genetically susceptible individuals frequently show subclinical intestinal inflammation and increased intestinal permeability even before the onset of clinical symptoms. [15, 16]

ANTI-INFLAMMATORY DIET IN CROHN'S DISEASE?

In the treatment of IBD patients, a patient-specific and carefully designed diet is an essential factor. This can prevent several nutritional deficiencies that are often seen in IBD patients. Until now, unfortunately, there is a lack of well-designed prospective intervention studies that examine specific dietary factors in CD. As a consequence, this evidence-based approach will be mainly based on retrospective (patient-reported) studies. [23] A review by Haskey *et al.* nicely summarizes some of the evidence-based dietary recommendations derived from literature for the maintenance of remission in IBD. [21] It is likely that a diet high in dietary fiber (especially from fruits and vegetables), low in refined carbohydrates and with limited red meat and saturated fats and low (*n*-6) poly-unsaturat-

ed fatty acids (PUFAs) intake will have a favorable effect.[24, 25, 17] Moreover, some authors suggest that certain dietary components can even have an additional anti-inflammatory effect in IBD patients. [18, 19] However, prospective studies evaluating the direct effect of a dietary intervention in IBD are scarce. [20, 21, 22] In this thesis, we have investigated the effect of riboflavin supplementation on the microbiota composition, inflammatory status, redox status and the symptoms and QoL in CD patients. In the *RISE-UP* study, we demonstrate anti-inflammatory and antioxidant effects of riboflavin. The *RISE-UP* study was designed as a proof-of-concept study to assess the potential of a dietary supplement to modulate various parameters of disease in a favorable manner. Due to the short intervention period (of only three weeks) and the short follow-up period, we did not assess the effect of this supplement on clinical relevant outcomes such as the frequency of exacerbations (or hospitalization). In the end, we would like to evaluate the effect of a complete (potentially beneficial) diet or a combination of multiple food components on clinical outcomes in CD. Therefore, our research group is currently preparing the ethical application of a follow-up intervention trial in the University Medical Center Groningen (UMCG), in which we aim to determine the clinical effect of an 'anti-inflammatory' diet in IBD patients. This 'Groningen anti-inflammatory diet' (*GrAID*) will be composed of a combination of many different food components based on an extensive review of the medical literature (evidence-based approach). This diet will be compared to a normal diet and diets with a vitamin B2/vitamin C supplement or a fiber (DM-Pectin) supplement. Participating patients will be instructed to adhere to this diet or food supplement group for a period of three months; this period is sufficient to also assess a potential effect on clinical outcomes such as frequency of exacerbations in CD patients. Altogether, these well designed prospective nutritional studies are very promising for improving disease outcomes for IBD patients.

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